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# Pre-Core Mutants of Hepatitis B Virus in Patients Receiving Immunosuppressive Treatment After Orthotopic Liver Transplantation

**Ulrike Protzer, Bernd Goergen, Uwe Hopf, Peter Neuhaus, Volker König, Karl-Hermann Meyer zum Büschenfelde, and Guido Gerken**

*1st Medical Department, Johannes Gutenberg University of Mainz (U.P., B.G., K.-H.M.Z., G.G.); Medical Department (U.H., V.K.), Department of Surgery (P.N.), Hospital Rudolf Virchow, Humboldt University of Berlin, Germany.*

Orthotopic liver transplantation (OLT) is a possible treatment for acute or chronic liver failure due to hepatitis B virus (HBV) infection, but reinfection of the graft can be a serious complication. The aim of this study was to monitor HBV markers, to analyse pre-core-/core-mutations as well as to identify the viral population causing reinfection after OLT, and to investigate the emergence or disappearance of these mutants in patients receiving immunosuppressive treatment.

Fifty-four pre-and posttransplant serum samples of 17 patients were analysed. All patients underwent OLT for HBV-related liver disease and had HBV-DNA before and after OLT. Total DNA was extracted from all sera and a 240 bp fragment comprising the pre-core region of HBV was amplified by polymerase chain reaction (PCR). Pre-core mutants of HBV were determined by direct sequencing of these PCR products and by sequencing of PCR clones.

Eight of 17 patients were infected with pre-core wildtype HBV before OLT (group A). Seven of eight patients of group A were reinfected by pre-core wildtype HBV after OLT. In one of eight patients in addition to wildtype HBV a mutant strain (nt. 1899 G → A) was detected. Nine of 17 patients were infected with pre-core mutant HBV before OLT (group B). Six of nine patients of group B were reinfected with the same mutant population; in one, an additional pre-core mutation emerged; two patients lost pre-core mutant HBV (nt. 1896 and 1899 G → A). In one of the latter two, a pre-core start-codon mutant (nt. 1816 G → T), not detectable before OLT, emerged, in the other a nt. 1897 G → A stop-codon mutant persisted. Five patients of each group were followed-up for more than 24 (25 to 58) months on immunosuppressive therapy. In all five patients of group A, pre-core wildtype of HBV persisted during long-term follow up. Two of five patients of group B were infected stably with a stop-codon HBV-mutant nt. 1896. In three patients, the nt. 1896 stop-codon mutant disappeared during immunosuppressive therapy. However, in one of the latter three, an HBV stop-codon mutant nt. 1897 persisted.

In conclusion, most patients who underwent OLT for HBV-related disease were reinfected with the same virus population that existed before OLT. In rare cases, new mutants emerged after OLT or preexisting mutants were lost. During long-term follow-up on immunosuppressive therapy, in the majority of patients pre-core mutants disappeared and wildtype HBV became the predominant virus strain. 

**KEY WORDS:** chronic hepatitis B, pre-Core-/Core-variants of HBV, liver transplantation, HBV-reinfection, evolution of HBV mutants

## INTRODUCTION

Orthotopic liver transplantation (OLT) is a possible treatment for patients with acute or chronic liver failure due to viral hepatitis [Perillo et al., 1993; Todo et al., 1991]. However, reinfection of the graft by persisting virus represents a potential complication in these cases. The clinical spectrum of reinfection ranges from mild persistent or self-limited hepatitis to fulminant hepatitis or to chronic active hepatitis with possible development of liver cirrhosis [Demetris et al., 1986]. Especially patients with chronic hepatitis B virus (HBV) infection are reinfected frequently after OLT. This reinfection seems to be associated with a high mortality rate [Bismuth, 1994; O'Grady et al., 1992; Samuel et al., 1993]. Recent studies have shown a correlation between the preoperative serum HBV concentration and the development of

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Address reprint requests to Prof. Guido Gerken, MD, 1. Med Klinik und Poliklinik, Johannes-Gutenberg-Universität Mainz, Langenbeckstr. 1, 55101 Mainz, Germany.

hepatitis in the graft [König et al., 1994; Samuel, et al., 1993]. In patients with persistent HBV infection undergoing OLT, application of anti-HBs immunoglobulin during and after liver transplantation can often prevent a clinically relevant reinfection of the graft [König, et al., 1994; Samuel, et al., 1993]. Application of immunosuppressive treatment in order to prevent rejection of the graft impairs T-cell response, which is normally responsible for the elimination of viral antigens [Calmus et al., 1990]. In addition, corticosteroids can directly stimulate HBV replication via a glucocorticoid-responsive element in the viral genome [Lau et al., 1992; Tiollais et al., 1985]. HBV usually replicates therefore at a high level in the immunosuppressed hosts after OLT.

HBV e-antigen (HBeAg) and core-protein (HBcAg) are encoded by the same open reading frame of the viral genome using two different mRNAs. These two antigens therefore share humoral and T-cell epitopes. HBeAg is secreted by wildtype HBV in abundance. But it has not been demonstrated if HBeAg plays a relevant role in initiating the infection or maintaining viral replication. It was shown that a membrane-bound form of HBeAg expressed on the surface of liver cells binds anti-HBe and is probably responsible for an antibody mediated immune response [Schlicht et al., 1991]. A peptide domain shared by HBeAg and HBcAg proved to be the major target for cytotoxic T cells [Bertoletti et al., 1993]. An increasing proliferation of T-cells to HBcAg and HBeAg was demonstrated at the time of clinical response during interferon treatment [Löhr et al., 1994].

HBeAg-negative variants of HBV have been found world-wide. The most frequent mutation nt. 1896 G → A generates a translational stop-codon at codon 28 of the pre-core/core-genome that abolishes synthesis of HBeAg [Carman et al., 1989]. Patients carrying this mutant are usually identified by ongoing HBV replication despite a negative serological test for HBeAg. Pre-core mutant HBV is sometimes—but not necessarily—linked to severe or rapidly progressive chronic hepatitis B [Brunetto et al., 1991; Goergen et al., 1994; Lai et al., 1994]. In the immunocompetent host with normal T-cell response, a pre-core defective mutant, unable to produce HBeAg, seems to be selected by the immune system of the host. It is particularly interesting to study patients on immunosuppressive treatment to elucidate the clinical relevance and evolution of pre-core mutant HBV.

The aim of our study was to monitor viral markers in patients who are infected with HBV before and after liver transplantation, to analyse pre-core/core-mutations and to identify the viral population causing reinfection. We also investigated the emergence and disappearance of pre-core mutant HBV in these HBV infected patients, who receive long-term immunosuppressive treatment.

## PATIENTS AND METHODS

### Patients

Fifty-four samples from 17 patients who underwent orthotopic liver transplantation (OLT) between September, 1988 and December, 1991 for HBV-related disease at the Liver Transplant Unit, Department of Surgery, Hospital Rudolf Virchow, Humboldt University of Berlin, Germany were examined. All the patients studied were reinfected with HBV after OLT. Only patients who proved positive for HBV-DNA before and after OLT were included. Sera from each patient were analysed before and after OLT. The first serum sample that proved positive for HBV-DNA after OLT, and follow-up samples at yearly intervals were subjected to sequence analysis. In four patients only one serum probe could be obtained after OLT (patients No. 10, 76, 87, 195).

Sixteen patients suffered from HBV-related liver cirrhosis. One patient had to be transplanted for fulminant hepatitis B with concurrent liver failure. Hepatocellular carcinoma was found incidentally in the cirrhotic liver of two patients by histological investigation of the explained organ (patients No. 172 and 212). Three patients had hepatitis Delta virus infection in addition to hepatitis B (patients No. 4, 86, 87) (Table II).

After OLT, all patients received immunosuppressive treatment in order to prevent graft rejection [König, et al., 1994]. 14 patients received a "standard" protocol with corticosteroids, cyclosporine A, azathioprine, and antilymphocytic antiserum. Three patients participated in an European multicentre trial and received FK 506 in addition to corticosteroids.

To prevent HBV-reinfection after OLT, all patients received anti-HBs immunoprophylaxis with human polyclonal anti-HBs hyper-immunoglobulin (anti-HBs Hlg, Hepatect®, Biotest, Germany) with an initial dose of 10,000 IU during the anhepatic phase. In four patients (patients No. 1, 4, 10, 11) a dose of 1000–2000 IU/day was applied only during the first postoperative week (short-term prophylaxis). The rest of the patients were continuously treated with anti-HBs Hlg in order to maintain an anti-HBs titre above 100 IU/I [König, et al., 1994; Samuel, et al., 1993]. Four patients (patients No. 1, 4, 11, 46) were additionally treated with interferon α 2b (Intron A®, Essex, Germany) for 3 months during the first postoperative year [Hopf et al., 1991]. However, interferon did not inhibit viral replication.

Patients were followed-up for 5 to 60 months during their clinical course (mean observation time 31.2 months), and 5 to 58 months after OLT (mean 28.8 months) (Table III). Three patients had to undergo five retransplantations; four patients died after 5 to 27 months. A histological follow-up was initiated by biopsy specimens taken during the transplantation procedure and repeated at regular intervals.

### Methods

***Viral markers.*** Sera from each patient were obtained before and directly after transplantation. During postoperative immunosuppressive and anti-HBs Hlg treatment follow-up sera were obtained in 3- to 12 monthly intervals. All sera were tested for HBsAg and HBeAg, antibodies to core (anti-HBc) and e antigen (anti-HBe) as well as to hepatitis Delta virus (anti-HDV). The titre of antibodies to HBV surface antigen (anti-HBs) was determined quantitatively. A second genera-

TABLE I. List of the Nucleotide Sequences of Oligonucleotides Used as Primers for PCR Amplification or Sequencing

| Primer | Sequence (5′ to 3′) | HBV Genome position[a] | Sense |
|---|---|---|---|
| Routine use PCR primers (preS spanning) | | | |
| MD 16: | GTC CTA GGA ATC CTG ATG | 187–170 | − |
| MD 19: | GGG TCA CCA TAT TCT TGG | 2816–833 | + |
| PreC spanning PCR primers (b = 5′-biotinylation) | | | |
| P1: | GGG GAG GAG ATT AGG TTA A | 1743–1761 | + |
| P2b: | AAG GAA AGA AGT CAG AAG | 1976–1959 | − |
| Sequencing primer | | | |
| Seq PreC: | TAT TAG GAG GCT GTA GGC | 1771–1788 | + |
| Mutation-specific PCR primers | | | |
| PreC Stop: | GTG CCT TGG GTG GCT TTA | 1879–1896 | + |
| PreC Wt: | GTG CCT TGG GTG GCT TTG | 1879–1896 | + |
| P3 | AGT GCG AAT CCA CAC TCC | 2287–2270 | − |
| Pre-core/core spanning PCR primers | | | |
| P4: | TTGTTCCCAAGAATATGGTGACC | 2840–2818 | − |
| P5: | GAGGACTCTTGGACTTTCAGC | 1661–1681 | + |

[a]All sequences are numbered according to Valenzuela, subtype adw (21).

TABLE II. Characteristics of the Patients Studied With Acute or Chronic Liver Failure Due to HBV Infection Before and After OLT*

| Patient No. | Group | HBV Precore mutations[a] (before OLT) | HBsAg pre/post OLT | HBeAg pre/post OLT | Histology of liver explant |
|---|---|---|---|---|---|
| 1 | A | wildtype | +/+ | −/+ | fulminant hepatitis |
| 11 | A | wildtype | +/+ | +/+ | cirrhosis |
| 46 | A | wildtype | +/+ | +/+ | cirrhosis |
| 159 | A | wildtype | +/+ | +/+ | cirrhosis |
| 195 | A | wildtype | +/+ | −/+ | cirrhosis |
| 202 | A | wildtype (+nt. 1896 G→A) | +/+ | −/− | cirrhosis |
| 212 | A | wildtype | +/+ | +/+ | cirrhosis + HCC |
| 227 | A | wildtype | +/− | +/− | cirrhosis |
| 4 | B | nt 1896 G→A + nt 1899 G→A + wildtype | +/+ | −/− | cirrhosis +HDV |
| 10 | B | nt 1857 G→T + nt 1896 G→A + nt 1899 G→A + nt 1897 G→A | +/+ | −/− | cirrhosis |
| 76 | B | nt 1896 G→A (+wildtype) | +/+ | +/+ | cirrhosis |
| 86 | B | nt 1896 G→A + wildtype | +/− | −/(+) | cirrhosis + HDV |
| 87 | B | nt 1896 G→A + nt 1899 G→A + wildtype | +/+ | −/− | cirrhosis + HDV |
| 95 | B | nt 1896 G→A | +/+ | −/− | cirrhosis |
| 99 | B | nt 1896 G→A | +/+ | −/− | cirrhosis |
| 143 | B | nt 1896 G→A | +/+ | −/− | cirrhosis |
| 172 | B | nt 1899 G→A | +/+ | +/+ | cirrhosis + HCC |

*(. . .) = Mixed population only detectable by mutation specific PCR; HCC = hepatocellular carcinoma; HDV = hepatitis Delta-virus superinfection.
[a]The point-mutation found is listed by the nucleotide position; all sequences are numbered according to Valenzuela, subtype adw (21).

tion tests for antibodies to hepatitis C virus (anti-HCV) was carried out. Commercially available enzyme-linked immunoassays were employed (Abbott Diagnostics, North Chicago, IL). Serum HBV-DNA was determined by commercially available solution hybridization assay with a detection limit of 10 pg/ml (Genostics, Abbott Diagnostics) [Gerken et al., 1991].

***Viral nucleic acid detection.*** Total DNA was isolated from 200 μl of serum by phenol/chloroform extraction after proteinase digestion as described in detail elsewhere [Gerken, et al., 1991]. Subsequently DNA was precipitated with ethanol, and after washing and drying, redissolved in 100 μl of distilled water. HBV DNA was detected by polymerase chain reaction (PCR). The pre-S1 and pre-S2 region of HBV was amplified from 10 μl (10%) of the obtained DNA solution. PCR amplification

TABLE III. Characterisation of the Patients After OLT and During Follow-Up*

| Patient no. | Group | Follow-up (months) | Histology of the graft/ course | HBV[a] Pre-core mutation (before OLT) | HBV[a] Pre-core mutation (post OLT) | HBV[a] Pre-core mutation (follow-up > 24 months) |
|---|---|---|---|---|---|---|
| 1 | A | 58 | ++ | wildtype | wildtype | wildtype |
| 11 | A | 42 | +/Rt | wildtype | 1899 G→A | wildtype |
| 46 | A | 47 | + | wildtype | wildtype | wildtype |
| 159 | A | 25 | +++ | wildtype | wildtype | wildtype |
| 195 | A | 5 | ++/† | wildtype | wildtype | 0 |
| 202 | A | 28 | + | wildtype | wildtype | wildtype |
| 212 | A | 23 | ++ | wildtype | wildtype | 0 |
| 227 | A | 7 | only MR | wildtype | wildtype | 0 |
| 4 | B | 58 | +++/2xRt | 1896 G→A<br>1899 G→A | 1896 G→A<br>1899 G→A | wildtype |
| 10 | B | 7 | fulminant hepatitis, +/2xRt, † | 1857 C→T<br>1896 G→A<br>1897 G→A<br>1899 G→A | 0<br><br>1897 G→A<br>— | |
| 76 | B | 27 | ++/† | 1896 G→A | 1896 G→A | 1896 G→A |
| 86 | B | 40 | only MR | —<br>1896 G→A<br>—<br>— | 1857 C→T<br>1896 G→A<br>1897 G→A<br>1899 G→A | 1857 C→T<br>—<br>1897 G→A<br>— |
| 87 | B | 22 | ++ | 1896 G→A<br>1899 G→A | 1816 G→T | 0 |
| 95 | B | 14 | +++ | 1896 G→A | 1896 G→A | 0 |
| 99 | B | 27 | + | 1896 G→A | 1896 G→A | 1896 G→A |
| 143 | B | 28 | ++ | 1896 G→A | 1896 G→A | wildtype |
| 172 | B | 19 | +++/† | 1899 G→A | 1899 G→A | 0 |

*Clinical course, follow-up time, and sequencing results are shown. HBV pre-core mutations found after OLT and during long-term follow-up of more than 2 years after OLT are compared to those found before OLT. Severity of hepatitis defined by graft histology is recorded. Typical features of fibrosing cholestatic hepatitis were not described histologically in any of the patients studied.
[a]The point-mutation found is listed by the nucleotide position; all sequences are numbered according to Valenzuela, subtype adw (21); Rt = retransplantation; † = died; 0 = follow-up < 24 months; Histology: + = mild, ++ = moderate, +++ = severe hepatitis; MR = mesenchymal reaction.

was carried out in a 100 µl volume containing 1 × PCR buffer (50 mM KCl, 1,5 mM $MgCl_2$, 10 mM Tris Cl, pH 8,3), 250 µM of each nucleotide (Boehringer Mannheim, Mannheim, Germany), 20 pmol of each primer (Table I) and 2 U of Taq polymerase (Gibco BRL Life Technologies, Paisley, Ireland). The following temperature profile was used: cycle 1: 5'/94°C, 1'/60°C, 1'/72°C; cycles 2 to 39: 1'/94°C, 1'/55°C, 1'/72°C; cycle 40: 1'/94°C, 1'/55°C, 4'/72°C. 5% of the amplification product was analyzed by electrophoresis in an ethidium bromide stained 1,5% agarose gel. This PCR [Gerken et al., 1991] is well established in our laboratory for routine use (detection limit 10 fg HBV-DNA/ml serum) and proved to be more sensitive than the PCR with pre-core/core primers described in nucleic acid sequencing.

***Nucleic acid sequencing.*** For the sequencing reaction, HBV DNA was amplified by 40 PCR cycles as described above incorporating a 5'-biotinylated downstream primer into the amplification product. Primers spanning part of the HBV preC/C genome (Nt. 1743-1976 according to [Valenzuela et al., 1980]) were used (Table I) as recently described in detail [Goergen, et al., 1994]. The biotinylated amplification product was bound to streptavidin-coated, magnetic micro-spheres (Dynabeads M-280, Dynal, Oslo, Norway) and denatured [Uhlen, 1989]. Fifty percent of this solid-phase bound single stranded DNA were sequenced directly according to the dideoxynucleotide chain termination reaction of Sanger et al. [1977] using T7-polymerase (T7-Kit, Pharmacia Biotech Europe GmbH, Freiburg, Germany) and $^{35}S$ dATP (Amersham Life Technologies, USA). The sequencing products were analysed by electrophoresis on a wedge-shaped, denaturing 12% polyacrylamide gel and subsequent autoradiography. Each amplification and sequencing experiment was carried out twice in order to minimise false results due to nucleotide misincorporation by Taq polymerase during PCR amplification. All sequences were numbered according to *Valenzuela*, subtype adw [Valenzuela et al., 1980].

***Mutation specific PCR.*** In addition to sequence analysis, an amplification refractory mutation detection system was applied as a fast, non-radioactive and even more sensitive method to detect the frequent stop codon mutation at nt. 1896 G → A as recently published [Goergen et al., 1994]. This mutation specific PCR (msPCR) was carried out in a 50 µl volume using two different upstream primers with the 3'-end at nt. 1896 that differ only in this last 3'-nucleotide (Table I). One primer exactly matches the wildtype sequence, the other matches the stop-codon mutation. Chain elongation occurs only

when the virus strand is complementary to the 3′ end of the according primer. To avoid false-positive results caused by primer mismatches PCR was run under most stringent reaction conditions (10 pmol of each primer, 75 μM of each nucleotide, hot start PCR) using the following temperature profile: cycle 1: 5′/94°C, 1′/68°C, 1′/72°C; cycles 2 to 26: 1′/94°C, 1′/66°C, 1′/72°C; cycle 27: 1′/94°C, 1′/66°C, 4′/72°C as described recently [Goergen, et al., 1994]. Cloned mutant and wildtype HBV-DNA was used as a specificity control.

***Cloning of PCR products.*** In selected patients No. 76, 86. and 87 PCR amplification of pre- and posttransplant sera was undertaken with primers spanning the whole pre-core-/core-genome (P4 and P5, Table I). A freshly prepared PCR product was ligated into vector pCR™-II (TA-Cloning Kit, Invitrogen Corp., San Diego, CA) and transfected subsequently into *E. coli* INVαF′ (One shot™ cells, Invitrogen Corp.) by the use of standard cloning techniques. Ten to fifteen individual clones from each PCR reaction were sequenced. DNA was prepared from an aliquot of *E. coli* cultured in LB-median by boiling for 5 minutes. 10 μl of this suspension were amplified by PCR (P1, P2b, Table I) and sequenced according to the protocol described above.

## RESULTS

### HBV Markers Before and After OLT

HBsAg and HBV-DNA were positive in all patients before OLT. After OLT, HBsAg was found in 15 patients either immediately or up to 24 months after OLT (Table II). Patients No. 4 and 11 lost HBsAg again after retransplantation for a short time. Two patients (No. 86 and 227) were constantly HBsAg negative after OLT, but were positive for HBV-DNA. The HBeAg/anti-HBe status of the patients before and after OLT is shown in Table II. In patient No. 86, HBeAg-status after OLT could not be determined definitely, because HBeAg and anti-HBe proved repeatedly borderline positive.

In nine of the 17 patients serum HBV-DNA was detectable by hybridization before OLT. In the remaining eight patients, HBV-DNA was detected before OLT by PCR with pre-S and pre-core/core primers. After OLT, sera of 16 of the 17 patients proved positive for HBV-DNA by hybridization technique. In the remaining patient, HBV-DNA was only detectable by PCR. Post transplantation, 15 of the 17 patients showed elevated liver enzymes. Liver histology in 15 of the 17 patients confirmed hepatic inflammation of the graft. Patients No. 86 and 227 had normal histology of the graft. Patients No. 86 and 227 proved consistently negative for HBsAg on continuous anti-HBs Hlg treatment. Patient No. 86 also had normal liver enzymes.

### Sequencing Analysis of the Pre-Core/Core Region of HBV Before and After Transplantation

Due to possible discrepancies between the occurence of HBV pre-core mutants and the HBeAg/anti-HBe status [Naoumov et al., 1992; Thomas et al., 1991], we divided the patients into two groups according to the HBV pre-core sequence determined prior to OLT (Table II, Fig. 1). Group A included eight patients who only carried the HBV pre-core wildtype prior to liver transplantation. Group B consisted of nine patients carrying pre-core mutants prior to OLT.

***Group A (pre-core wildtype HBV before OLT).*** In group A, in seven of the eight patients no pre-core variants were detected before and after OLT. In patient No. 11, a mutant virus strain with a point mutation at nt. 1899 G → A was observed in addition to the wildtype virus, that causes an aminoacid exchange at aa 29 (Fig. 1). However, this mutation disappeared again during further observation (Table III). In two patients of group A (No. 202 and 227), anti-HBe persisted although they were reinfected with predominantly wildtype HBV. A mixed HBV population of predominantly wildtype and a minor percentage of nt. 1896 stop-codon mutant HBV was identified in patient No. 202 by mutation specific PCR. In patient No. 227, mutant HBV could not be detected.

***Group B (pre-core mutant HBV before OLT).*** All patients with HBV mutants carried at least one of the most frequent mutations affecting the pre-core genome of HBV at nt. 1896 (codon 28) or nt. 1899 (codon 29) (Fig. 2). Thus the prevalence of the stop-codon-mutation at nt. 1896 in 17 patients undergoing OLT was 47% (eight of 17), the prevalence of the mutation at nt. 1899 was 24% (four of 17). Four of the nine patients of group B carried a mixed viral population with detectable HBV wildtype strains besides mutant HBV (Table II).

In group B, six of the nine patients carrying HBV pre-core mutants were reinfected with the same virus population as detected before OLT (Fig. 1). In three patients, different HBV mutants were found after OLT.

Patient No. 87 proved constantly HBeAg negative/anti-HBe positive after OLT, although the HBeAg-negative stop-codon mutant HBV was overgrown by wildtype virus. Sequencing of PCR clones in this patient showed the stop-codon mutation nt. 1896 G → A in eight of 10 clones before OLT but only in three of 10 clones after OLT. Interestingly, after OLT a rare pre-core start-codon mutation at nt. 1816 was found in three of 10 clones with otherwise wildtype pre-core sequences (Fig. 3). A mutation nt. 1816 G → T also inhibits the translation of a pre-core/core precursor protein and thus abolishes synthesis of HBeAg.

Patient No. 86 harboured stop-codon mutant HBV (nt. 1896 G → A) prior to OLT in 10 of 10 analysed clones. After OLT, the stop-codon mutation at nt. 1896 G → A was still present, but additional mutations were found: a stop-codon mutation nt. 1897 G → A and point mutations at nt. C → T and nt. 1899 G → A (Table III). 47 months after OLT mutations at nt. 1896 and nt. 1899 were no longer detectable (zero of ten clones), whereas HBV with mutations at nt. 1897 and nt. 1857 persisted during the entire observation time (three of 10 clones). A second patient (No. 10) also lost the stop-codon mutant HBV (nt. 1896 G → A and nt. 1899 G → A) after OLT,

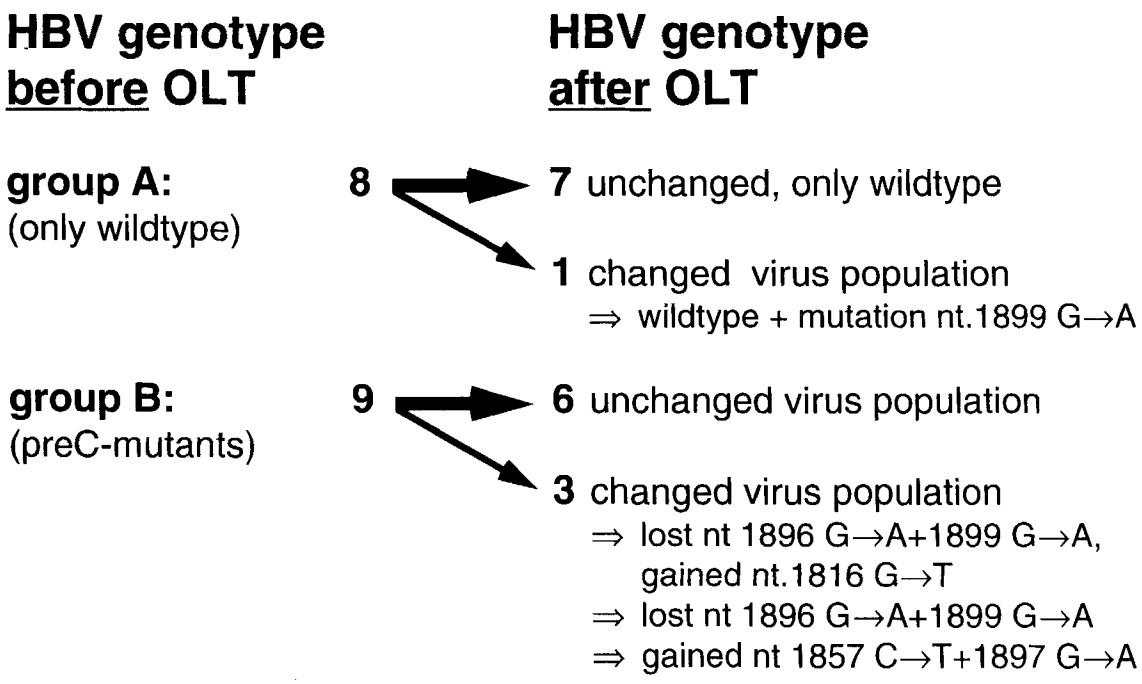


Fig. 1. Comparison of the hepatitis B virus population before and after liver transplantation. Patients of group A were infected with precore wildtype and patients of group B with precore mutants of hepatitis B virus before transplantation. In two of the three patients of group B, in whom virus population changes, precore wildtype HBV became predominant over the nt. 1896 stop-codon mutant virus (see text). In one patient, a new viral strain could be identified, in another patient, additional precore mutations were detected.

while HBV carrying mutations at nt. 1857 C → T and nt. 1897 G → A persisted during the follow-up period of 7 months (Table III).

### Long-Term Follow-Up During Immunosuppressive Therapy (>24 months)

Long term follow-up after OLT was performed in five patients each of group A and B on immunosuppressive treatment for more than 2 years. Based on the sequencing results, four patients (No. 1, 46, 159, 202) on long-term follow-up were reinfected only with wildtype HBV, whereas two patients (No. 76 and 99) were reinfected only with nt. 1896 stop-codon mutant HBV. Four patients (No. 4, 11, 86, and 143) were reinfected with a mixed population of wildtype and pre-core mutant HBV (Table III). In four patients (No. 1, 4, 11, 46), who received interferon α 2b treatment, no significant change of the HBV population was induced by this treatment.

***Group A (pre-core wildtype HBV before OLT).*** Pre-core wildtype HBV was present during long-term follow up for up to 58 months on immunosuppressive treatment in all patients of group A (No. 1, 11, 46, 159, 202). In patient No. 99, a virus strain with a mutation at nt. 1899 G → A emerged for a short time directly after OLT, but was lost again during further follow-up. In the remaining four patients no pre-core mutants could be detected by sequence analysis (Table III, Fig. 4).

***Group B (pre-core mutant HBV before OLT).*** In two of the five patients (No. 76 and 99) of group B, the mutant HBV population did not change. Sequence analysis showed only nt. 1896 stop-codon mutant virus before OLT and during follow up of 27 months on immunosuppressive therapy. Patient No. 76 constantly proved HBeAg positive in spite of an infection with predominant stop-codon mutant HBV. In this patient, mutation specific PCR detected repeatedly a small amount of wildtype virus in addition to the nt. 1896 mutant. Sequencing of PCR clones revealed wildtype virus in only one of 10 clones before and in none after OLT.

Patients No. 4, 86, and 143 had been infected by stop-codon mutant HBV prior to OLT. All three patients lost the virus strain with the nt. 1896 stop-codon mutation under long-term immunosuppression. In patient No. 86, a mixed HBV population consisting of wildtype virus in addition to a virus strain with mutations at nt. 1897 G → A and nt. 1857 C → T persisted. In patients No. 4 and 143, the mutant strains were overwhelmed by wildtype strains (Table III, Fig. 4).

## DISCUSSION

The course of HBV infection in patients undergoing liver transplantation is particularly interesting to elucidate the clinical and biological significance of HBV mutants. Nucleotide sequence analysis was administered

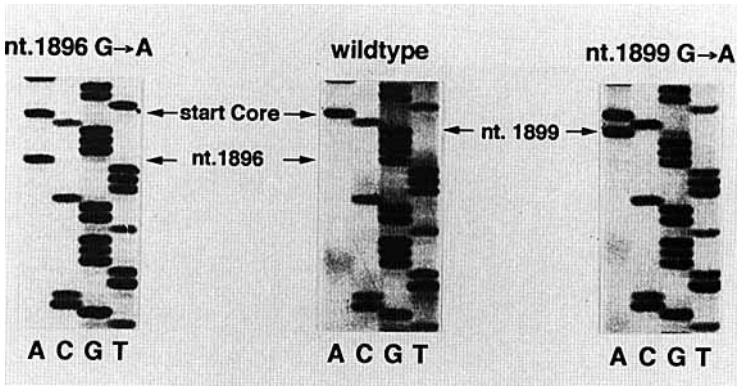


Fig. 2. Variability of the hepatitis B genome. Nucleotide sequencing results show frequently observed HBV precore mutations. A point-mutation at nt. 1896 G → A causes a stop codon 28 and abolishes HBeAg production. A point-mutation at nt. 1899 G → A causes an aminoacid exchange codon 29.

to search for homology between the original virus, identified prior to transplantation, and that causing reinfection post transplantation. We determined the composition of the viral population and studied the evolution of pre-core mutant HBV in an immunocompromised host during immunosuppressive treatment after OLT.

In our study, including 16 patients who underwent liver transplantation because of endstage liver disease and one patient with fulminant hepatic failure due to HBV infection, we found a high prevalence of pre-core/core mutants of HBV. The prevalence of a stopcodon mutation at nt. 1896 was 47%, the prevalence of mutation at nt. 1899 was 24% prior to OLT. A major percentage of HBV pre-core mutants existed as a mixed population with the wildtype virus. A similar observation was made by Lai et al. [1994] who reported that viral mutants are enriched during long-lasting HBV infection with ongoing viral replication. The HBeAg/anti-HBe status of a patient does not necessarily predict the existence of viral mutants [Naoumov, et al., 1992]. One of the patients (No. 76) was HBeAg-positive/anti-HBe-negative, although HBeAg-negative stop-codon mutant HBV made up more than 90% of the viral population in the patient's serum. Recent studies detected persistent HBV pre-core wildtype strains producing HBeAg in liver tissue specimens of such patients [Dienes et al., 1994; Lai, et al., 1994].

We were able to show that the majority of patients are reinfected after OLT with the same virus population of HBV that was present before OLT. New pre-core mutants occured in only three of the 17 patients after OLT. In one of our patients (No. 87), a new viral strain was found after OLT (Table III, Fig. 3). A similar observation has recently been described by Laskus et al., [1994]. Sequencing of PCR clones gave no evidence that the newly found strain preexisted before OLT. However, although ten clones each before and after OLT were analysed, a rare virus strain which represents less than 10% of the viral population could have been missed. An infection during or after OLT with a different strain of HBV has to be taken into consideration, but the appearance of a new mutation can not be excluded. Reinfection of the liver graft after OLT is most likely to occur from extrahepatic reservoirs of HBV, e.g., infected lymphocytes [Jiang et al., 1994]. The occurrence of HBV-DNA in extrahepatic tissues has been shown by in situ hybridisation [Mason et al., 1993]. Chazouilleres et al. [1994] were the first to demonstrate that infection with HBV can also be acquired with liver transplantation.

The outcome of HBV infection reflects a balance between the host's immune response and the ability of the virus to escape it. HBV replicates via an intermediate RNA pregenome [Tiollais, et al., 1985]. This explains the high rate of nucleotide misincorporation during transcription and resulting variations of the viral genome [Carman et al., 1992]. Only a portion of these variants will survive, provided that the mutation confers an advantage over the pre-existing virus strain. Since the HBeAg seems to bear immuno-modulation function [Ferrari et al., 1993; Thomas and Carman, 1991], we investigated mutations in a region of the HBV genome that codes for the pre-core/core precursor protein which is further processed to HBeAg/ This was done in patients receiving immunosuppressive treatment. Our results show that during immunosuppressive treatment the same pre-core mutants are detected as previously found in the immunocompetent host [Kremsdorf et al., 1993; Lok et al., 1994; own observations].

On the other hand, in four of eight patients carrying a stop-codon mutant nt. 1896 either before or after OLT, the pre-existing mutant disappeared after 7 to 52 months on immunosuppressive treatment and was replaced by a pre-core wild-type virus strain. We suggest that the stop-codon mutant nt. 1896 G → A no longer has a significant advantage over the wildtype virus once adequate T-cell response by the host is lacking. In addition, wildtype HBV has to replicate more effectively during immunosuppressive therapy than certain pre-core mutants of HBV. The only pre-core mutation in this study, that became persistently detectable during immunosuppres-

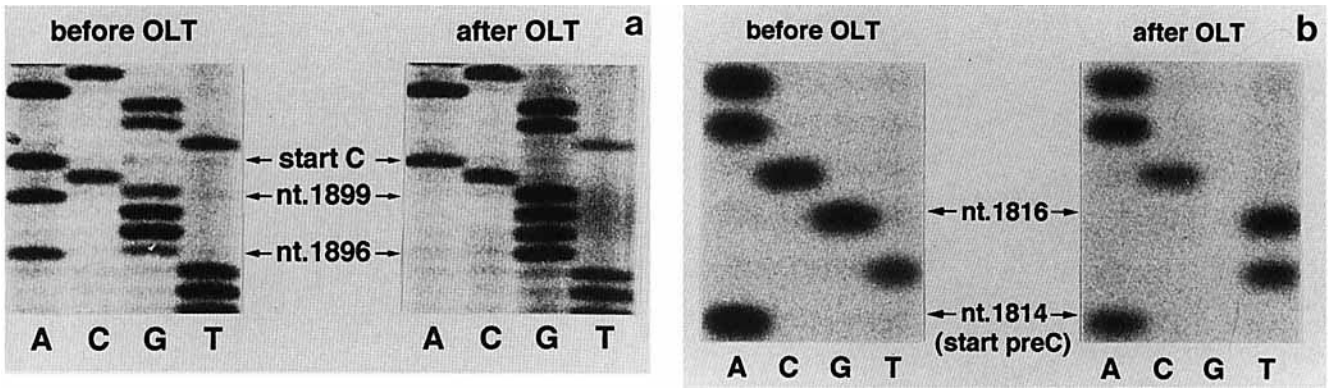


Fig. 3. Sequencing results of serum HBV-DNA of HBeAg negative/anti-HBe positive patient No. 87 before and after OLT. The stop codon mutation at nt. 1896 G → A detectable before OLT, accompanied by a mutation at nt. 1899 G → A, disappeared after OLT **(a)**. After OLT, a HBV-mutant with a pre-core start-codon mutation at nt. 1816 G → A had emerged that prevents production of HBe protein as well **(b)** (see text).

sive treatment, was a stop-codon mutation nt. 1897 G → A accompanied by a point mutataion nt. 1857 C → T. Vanishing of certain pre-core mutants of HBV during long-term immunosuppressive therapy has not been reported before to our knowledge. The only two other studies, that investigated pre-core mutants of HBV after OLT, either terminated the follow-up at 24 months [Laskus, et al., 1994], or only analysed one postoperative serum per patient [Angus et al., 1995].

In general, the number of HBV genomes in HBeAg-negative sera seems to be much lower than that in HBeAg-positive sera. In longitudinal follow up of chronically infected patients, anti-HBe seroconversion is frequently accompanied by a reduction of hepatitis B virus replication at the time of emergence of a pre-core stop codon mutant [Goergen, et al., 1994; Lai, et al., 1994; Zarski et al., 1994]. This indicates that HBeAg-negative HBV mutants replicate less effectively [Blum et al., 1991; Mason et al., 1993; Omi et al., 1990]. However, some virus strains with mutations in the core and in the X gene in addition to the pre-core stop codon are described to replicate highly effectively [Ogata et al., 1993]. Coinfection of ducklings with pre-core mutant and wildtype duck hepatitis B virus resulted in overgrowth of the precore mutant by wildtype virus in the duck model [Chuang et al., 1994]. The study aimed at giving insights into the mechanisms of perinatal transmission of HBV-infection, but the immune status of the newborn ducklings is in some aspects comparable to the situation we observed. Consistent with the obviously lower replication rate of most pre-core mutants *in vivo*, the virus strain carrying mutations at nt. 1896 and nt. 1899 was overgrown in the majority of our patients. Whether the replication rate of precore mutants is dependent on genomic changes or loss of HBeAg remains unknown.

A mutation in the pre-core/core genome of HBV might also affect an RNA sequence referred to as the "ε" encapsidation signal. The "ε" signal serves as a signal for two biochemically distinct events, RNA packaging and reverse transcription. Therefore mutations in this region may influence viral replication [Bartenschlager et al., 1993; Junker-Niepmann et al., 1990; Tong et al., 1992]. In consequence, only two out of ten theoretically possible stop-codon mutations in the pre-core/core region are found to occur naturally (nt. 1896 or 1897 G → A) [Kremsdorf and Brechot, 1993; Lok, et al., 1994; Tong, et al., 1992]. The only pre-core mutant HBV strain that emerged and persisted during immunosuppressive treatment in our study, carried a stop-codon mutation at nt. 1897 G → A accompanied by a point-mutation at nt. 1857 C → T. The stop-codon mutation at nt. 1897 G → A abolishes the production of HBeAg. The accompanying mutation at nt. 1857 C → T stabilises the stem-loop structure of "ε" again. This possibly accounts for a replicatory advantage and causes the overgrowth of HBV wildtype or other mutants by the observed mutant virus strain. A similar advantage was stated by Lok et al., [1994] for mutations at nt. 1896 G → A and nt. 1899 G → A. However, in our study in the setting of immunosuppression, an advantage for mutations at nt. 1896 and at nt. 1899 could not be confirmed.

In conclusion, reinfection after OLT occurs in most patients with the same virus population existing before OLT. In rare cases, new mutants are detected or preexisting mutants disappear directly after OLT. During follow-up on immuno-suppressive therapy, half of the patients lost the preexisting stop-codon mutation nt. 1896 in spite of an ongoing HBV wildtype infection. This precore stop-codon mutant of HBV, unable to produce HBeAg, seems to be selected by the immune system of the host. In a host who is on immunosuppressive treatment, this mutant seems to lose its advantage over wildtype virus. In our study, only an HBV strain with a stop-codon mutation nt. 1897, accompanied by a mutation nt, 1857 that serves to stabilize the RNA encapsidation signal of HBV, could persist during long-term follow up on immunosuppressive therapy and could develop to the predominant virus strain.

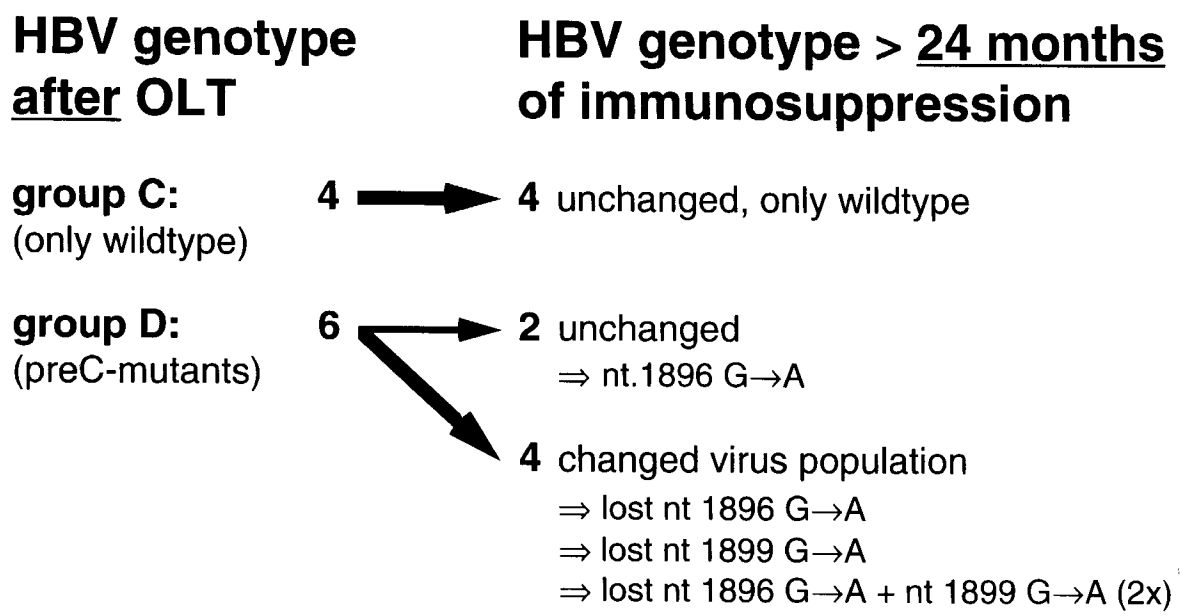


Fig. 4. Long-term follow-up of the patients receiving immunosuppressive therapy for more than 24 months after liver transplantation. The follow-up of patients carrying only wildtype HBV or mutant HBV directly after OLT is shown. During follow-up in these immunosuppressed patients, who lack adequate immune response to viral antigens, the virus strains, that harbour the stop codon mutation at nt. 1896 G → A, seem to lose its advantage.

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